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Anti-infammatory agents and their effect upon inflammation in the suprapatellar bursa of rabbits

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H. TERRY WEPSIC

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ANTI-INFLAMMATORY AGENTS AND THEIR EFFECT UPON INFLAMMATION IN THE SUPRA-PATELLAR BURSA OF RABBITS

H. TERRY WEPSIC

A thesis presented to the faculty of the Yale University School of Medicine in candidacy fo the degree of Doctor of Medicine.

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(1965)

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INTRODUCTION

I. The acute inflammatory response

A. Historical considerations

A definition of the process of inflammation has been made by Burden and Sanderson. "The process of inflammation is the succession of changes which occurs in a living tissue when it is injured, provided that the injury is not of such a degree as at once to destroy its structure and vitality."[36] The important part of this definition is that first inflammation is not a state but rather a process and secondly that this process involves a succession of changes occurring at the site of tissue injury.

Classically inflammation is thought of as a reaction to bacteria and other living agents which can be injurious to viable tissue. We must remember, as Robbins points out in his general textbook on pathology, that the cause of inflammation may include many non-living agents such as heat, cold, radiant energy, electrical or chemical stimuli, and simple mechanical trauma.[85]

The inflammatory response in vertebrates is composed of a complicated series of physiologic and morphologic adjustments involving chiefly the blood vessels, the fluid and cellular components of the blood, and the surrounding connective tissue. Examination of many of the vascular and fluid adjustments which occur in the inflammatory tissues was not undertaken until technical advancement of the twentieth century made these problems easier to investigate. The cellular reaction was, however, investigated by Eli Metchnikoff, a non-medical biologist of the nineteenth century.

Metchnikoff was the first person to point out that the exudation of cells seen in the acute inflammatory situation was of great importance.[70] He traced the essential continuity of the phagocytes during the evolutionary stages from protozoa, through the primitive metazoa and sponges to the mesenchymal phagocytic cell systems of higher animals.[53] Metchnikoff stoutly maintained that the cells that appeared during the inflammatory process were the chief protection of the animal against invasion. This view did not go unchallenged and potent arguments were advanced that the main protective function resided in the liquids of the blood, the so called humoral view.[36]

B. The sequence of events in the acute inflammation

Following local acute injury several changes occur at the site where the blood vessels enter the inflammation. These changes are dependent upon two mechanisms. First, there is stimulation of nerves which causes a local axon reflex. This results in a transient vasoconstriction and hence local anemia. Following this vasoconstriction, which is initiated by the axon reflex arcs, there is a compensatory arteriolar dilatation with an increased rate of blood flow through the arterioles, capillaries, and venules. This dilitation is believed to be dependent on both nervous and humoral factors.[36] The exact nature and site of origin of these humoral agents are still unknown, but it is generally believed that histamine may be among those present.[61]

The other agents besides histamie which have been implicated include serotonin, certain peptides,[33, 69, 95] globulins[68, 70, 87,] nucleosides and nucleotides.[101, 96] The vasodilatation, which is prolonged by the presence of humoral vasoactive amines, leads to a sludging or stasis of blood flow. In addition to causing vasodilitation, the chemical mediators alter the endothelial lining to increase the permeability. This increase in endothelial permeability and resultant outpouring of fluid into the inflammatory area is also associated with an increase in pressure due to the stasis of blood flow. The increase in capillary permeability included not only increased premeability to fluid and electrolyted but also the increased permeability to protein components of the plasma as well (globulin, albumin, fibrinogen). As more tissue exudate accumulates there is a concomitant increase in lymphatic flow. The lymphatice dilate because the fluid stretches collagen fibers attached to the outside of the endothelial walls and so holds them open against a pressure greater than that about them.[19] At the same time their permeability is altered much as isthat of the capillary endothelium. This increase in permeability causes the lymphatics to remove a larger amount of inflammatory exudate from the inflammed area.[2, 36, 53, 85]

Since inflammation is an active process, the events just described of arteriolar dilitation, stasis of blood flow, and extravasation of fluid are occurring spontaneously, each with its rate and with its own interrelationship to the other inflammatory events. This generality applies to all the changes which are seen in inflammation.

The other [illegible] which have been implicated 50, 51, 52, [illegible] include serotonin, [illegible], [illegible] 70, 87, [illegible] 35, 36 nucleotides and [illegible]. The [illegible], which is [illegible] by the [illegible] of [illegible], leads to a slowing of [illegible]. In addition to causing vasodilatation, the chemical [illegible] endothelial lining to increase its permeability. This increase in endothelial permeability and [illegible] of fluid into the [illegible] is also associated with an increase in pressure due to the [illegible]. The increase in capillary permeability involves not only increased permeability to fluid and [illegible] but also the increased permeability to [illegible] of the plasma as well (albumin, fibrinogen, [illegible]). As the tissue fluid accumulates there is [illegible] of lymphatic flow. The lymphatics [illegible] stretched [illegible] endothelial cells and [illegible] pressure greater than that [illegible].[11] At the same time their permeability is altered much as [illegible] capillary endothelium. This increase in permeability allows the lymphatics to remove a large amount of inflammatory exudate from the inflamed area.[9, 38, 39, 40]

Once inflammation is an active process, the events just described of arteriolar dilatation, slowing of blood flow, and extravasation of fluid are occurring simultaneously, each with its own [illegible] to the other inflammatory events. They [illegible] to all the changes which are seen in inflammation.

The Clarks (1932)noted that the first observable cellular change following tissue injury and the injection of foreign material was the adherence of leukocytes to the vascular endothelium.[19] This observation was a confirmation of earlier studies of Cohnheim (1882).[21] The reasons for this pavementing of the white cells have never been adequately established. Three possible explanations for the margination have been suggested. First, white blood cells may become adherent to the endothelium because of increased stickness of either the white blood cells or the endothelial cells lining the capillary. Second, margination of the white blood cells may be dependent upon some physical change in a particular site of the endothelial lining. Thirdly, the white blood cell attraction might be a response of the leukocytes to some chemotactic stimulus. Florey observed that the electron micrographs of vessels in which "sticking" is taking place showed that the leukocytes come into intimate contact with the inflammed endothelium, and that there is no electron dense material on the endothelial surface, or any other evidence that a "cement" is secreted to which the leukocytes adhere.[36] This margination which occurs is reversible if the inflammatory stimulus was a mild one and the endothelium can return to normal. Once in contact with the endothelium, cells are seen to migrate through endothelium into the area of the inflammatory exudate. The electron micrographs presented by Florey in his textbook of general pathology to illustrate this migration are incomparable, as is his description:

> ...the leukocyte puts our pseudopods. If one is put out near the cell junction it will force its way down the junction, and our present information is that passages of leukocytes through the wall are made through or at least very close to a junction... The pseudopod finding its way between these structures is sometime long and relatively tenuous. A number of leukocytes that have partially migrated may be held up in the periendothelial space, forming a ring outside the endothelium. After the leukocyte has traveled parallel to the vessel wall for a certain distance, a pseudopod eventually finds its way into surrounding connective tissue, tobe followed by the rest of the leukocyte......36

C. Chemotaxis

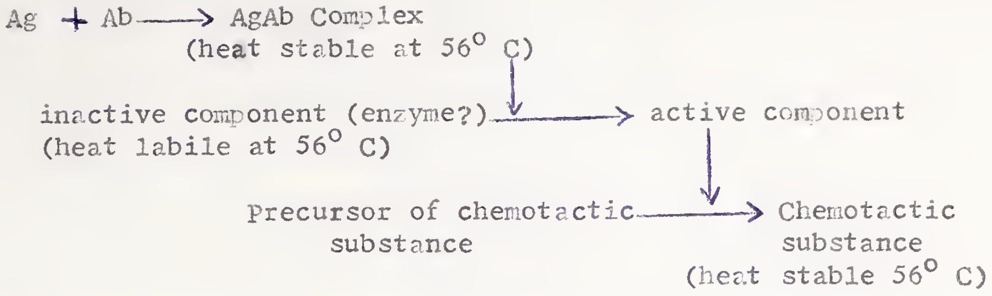
The mechanism by which leukocytes become attached to the capillary wall is not completely established. One of the possible explanations was that the white blood cell attraction might be a response of leukocytes to some chemotactic stimulus. It has been noticed since the time of Metchnikoff that polymorphonuclear leukocytes tend to accumulate around clumps of various sorts of foreign material (e.g. bacteria, starch grains etc) both *in vivo* and *in vitro*. Prior to Metchnikoff, Leber in 1888 labeled this effect of attraction for leukocytes "chemotaxis."[60] It has indeed been shown by many workers that the effect of leukocytic attraction is not merely due to the development of paralysis or of a "stickiness" of the leukocytes when random movement brings them close to the clumps of foreign material but that there is actual directional movement.[23,43,64, 65] Following these observations there was systematic investigation of the substances which could cause leukocytic migration. It was seen that proteins,[40] polysaccharides,[67] simple sugars,[40] and polypeptides[68] were all substance which attracted leukocytes. Although this work is open to technical criticism, there is no doubt that polymorphs tend to accumulate around clumps of foreign material.

Recently a new technique for the examination of chemotaxis was developed by Boyden.[13] This technique involves the use of a Perspex (lucite) chamber which is spearated into two components by a millipore filter membrane of such a pore size (e.g. 3u) that leukocytes cannot pass through except by active migration. The cells to be examined, usually rabbit polymorphonuclear leukocytes, can be placed on one side while the substance to be tested is placed on the other. Boyden noted that chemotactic leukocyte movement seemed to be dependent upon a group of similar chemical substances. Some of these substances such as amino acids and polysaccharides could be associated with tissue breakdown seen in the acute inflammation. The suggestion might therefore be made that this selective process of cell attraction, chemotaxis, might be very much like cell recognition of foreigness which occurs in antibody production. The movement of the phagocytic cells is a response to the presence in their enviroment of macromolecules which differ in structure from those normally present in the host. These macromolecules may be very similar to or even of the same family of molecules that give rise to antibody formation.[13] Boyden noted, in general, that the further the biological source of the test material is phylogenically removed from the rabbit, the more active is the material likely to be as a chemotactic agent for rabbit polymorphs. This same statement could be made of the relative antigenic capacity of various related proteins.

Boyden's main observations were with mixtures of antibodies and antigens. He noted that human serum albumin, which has little or no chemotactic activity when tested in medium containing normal serum, caused marked leukocytic migration in the presence of rabbit antibodies to human serum albumin. At first

it was though that the antibody-antigen complexes were exerting a direct effect of the white blood cells. It was, however, noted that the chemotactic effect was strongest when the maximum precipitate occurred, in the region of antigen-antibody equivalence. This was indeed contradictory for in the region of antibody-antigen equivalence there would be less complex coming in contact with the cells than in such mixtures as would be in the regions of antigen excess. Subsequent investigation showed that when antigen-antibody mixtures in the equivalence zone were incubated in normal serum at 37° C and then centrifuged, the resultant supernatant was strongly chemotactic. Since the supernatant was "chemotactically" active it was suggested that the agent(s) acting on the leukocyte might not be the antigen-antibody complex itself but rather some by-product of antigen-antibody interaction.

This chemotactic activity was not abolished by incubation at 56° C. From this work and other experiments three general conclusions were drawn. First, leukocytes are capable of responding to chemotactic stimulus by active directional migration in the presence of inactivated as well as fresh serum. Secondly, the interaction of antigen-antibody results in the production of heat-stable chemotactic substance(s). Thirdly, that the chemotactic substance is not produced when antigen-antibody are allowed to interact in heat inactivated serum. It appears that antigen-antibody complex (heat stable) activated a non-specific, heat labile serum system (complement?) with the result that "polymorphonuclear chemotactic principle" (heat stable) is produced. This can be diagrammed as shown below.



It was previously suggested that chemotactic particles appeared to be of the same or similar family as the foreign substances which are antigenic in many species. If "natural" antibodies existed in the animal to many of these foreign substances which are being investigated as possible causes of chemotaxis, than the antigen-antibody interaction could lead to the formation of "chemotactic substance" and chemotaxis would therefore result. This could also occur in animals where "very foreign" bacteria which present a large number of different and unfamiliar foreign determinant groups could be chemotactic in normal serum because natural antibodies provide levels high enough for antigen-antibody interaction.

Boyden has gone even further and has proposed that it is not actually the "chemotactic substance" which cause the movement of the white blood cells but rather a difference in the concentration of this substance around particles. This hypothesis was possible suggested by McCutcheon's work.[64,65,66] McCutcheon showed that collodion particles are strongly chemotactic for polymorphs. He suggested, after ruling out attraction due to electrical charge, that the collodion particles absorb certain components from the serum and that polymorphonuclear leukocytes are attracted toward areas around particles where the concentration of these substances is lower than elsewhere in the medium.

The examination of the chemotactic properties of bacteria was done by Coman et al (1939).[22] The serum of animals immunized to a particular bacterial species did not increase the chemotactic response to the bacteria as compared to serum from normal unimmunized animals. Immunization seemed to be of little value with regard to the chemotaxis of bacteria. Boyden explains this by suggesting that perhaps in the system used by Coman et al chemotactic principle, so that no difference in concentration could exist around any one particle.

Although many investigations have examined various substances for their chemotactic capacity, it is impossible and of little value to review each substance examined. One system which is important is that of tissue products. Harris (1953) observed that dead granulocytes, crushed and autolyzed tissue, enzymatic digest of tissue, and exudates produced by intraperitoneal injection of isotonic saline were not chemotactic.[42] Hurley and Spector (1961) disagree with this on the grounds of experiments done with their <u>in vivo</u> system. They noted that rabbit liver homogenates, injected intradermally, caused only slight leukocytic migration into the area of injection, unless the homogenate is first incubated with normal rabbit serum. If this was done there is marked leukocytic response.[54] This further substantiated Boyden's hypothesis that interaction of a substance (tissue products) with serum results in the liberation of a "chemotactic substance" which causes chemotaxis by its particular distribution about the particles in solution.

Since chemotaxis has been investigated for well over sixty years, many results, theories and counter theories have arisen. What has been attempted is not a review of all the literature on this field, but rather a review of a unified hypothesis presenting

one possible explanation for chemotaxis which is compatible with experiment work presented by this paper.

D. Phagocytosis

Thus far in the discussion we have been dealing with events which occur during the acute process of inflammation. Such a discussion would be of little value if it did not investigate the process of phagocytosis. Without the actual process of cells ingesting foreign material (phagocytizing), the other events of inflammation would be of little actual value.

As mentioned previously it is to Metchnikoff that we owe the establishment of the process of phagocytosis firmly as an important defense mechanism. There still remains many unanswered questions concerning the basic fundamentals associated with phagocytosis. Excellent reviews on the suject of phagocytosis are given by Mudd et al[73] and Suter.[99]

Throughout the paper references have been made somewhat casually and interchangeably to leukocytes, and to polymorphonuclear leukocytes. It is important to investigate the type of cells most commonly involved in phagocytosis. Often the capacity to phagocytize is not appreciated although it occurs in most cell types in the mammalian body.[12] Phagocytosis has been reported not only in the white blood cells associated with the circulating blood but also has been reported in fibroblasts, renal tubular cells, liver parenchymal cells, endodermal cells of the intestine, pigment cells of the retina, smooth muscle cells, skeletal muscle cells, certain epithelial cells,[82, 93] and possible platelets.[97, 100] Of the mature leukocytes, the granulocytes and monocytes are potentially phagocytic. The

The polymorphonuclear leukocytes are more phagocytic than eosinophils and basophils. Some disagreement exists as to whether the mature lymphocyte is or is not phagocytic in the same sense as the other while blood cells.[6, 83] The younger mature granulocytes with one or two nuclear lobes are the most effective phagocytes.[86] The phagocytic activity was shown to be decreased in the granulocytes from infants,[63] myeloid leukemia patients,[15] and in animals which have various vitamin deficiencies[72, 76]. It has also been noted that the blood from patients on corticotropin and cortisone therapy has decreased phagocytic activity.[25] The ability of leukocytes to phagocytize is also affected by temperature. Below 15° C there is relatively little phagocytic activity; the activity increases with the approach to 37° C but above 40 to 42 °C it again diminishes.[123]

Besides the phagocytic cell itself many circumstance exist which can modify phagocytosis. These may be divided into those factors which are associated with the particles phagocytized and factors in the enviroment. Concerning those factors associated with particles, it has long been known that certain antiphagocytic factors are produced by bacteria and actually may inhibit their ingestion. This capacity is frequently associated with bacterial virulence, or the ability to parasitize and invade animal tissues. Examples of this antiphagocytic capacity include certain pneumococcus capsular polysaccharides,[117] somatic "O" antigens on the surface of many gram negative species; and of the exotoxins and leukocidins of many gram positive agents. Non bacterial substances such as mucin[115] and certain surface active agents[7] may also be antiphagocytic. On the other hand factors exist which promote phagocytosis. One of these factors has been termed "opsonin." This term was first introduced by Wright and Douglas (1903, 1904)

to describe the substances responsible for the heat labile (56° C) property of normal mammalian serum of rendering certain bacteria liable to phagocytosis by leukocytes.[121, 122] Subsequently it was shown by Bullock and Western (1906) that the treatment of normal serum with tubercle bacilli, which were subsequently removed by centrifugation, resulted in the removal of the opsonic power of the serum for the tubercle bacillus, while opsonic activity for the staphlocci remained almost unaltered. The converse of this experimental situation is also true.[16] This observation, coupled with others, leaves little doubt that the opsonins of normal serum are as specific as antibodies.[46, 62, 74] Complement is necessary for the action of opsonins explaining their apparent heat labile properties.[24] The main common denominator in opsonic action is the ability of opsonins to coat the surface of the microbe in order to decrease the surface electrical potential and promote adhesion to other such particles (agglutination), and to the surface of the phagocyte.[53] The most active opsonins are the specific antibodies, occurring in the serum of immunized objects, which combine with the antigenic chemical components (the polysaccharides or polypeptides) of the bacillary capsules. The activity of other opsonins which are not specific antibodies is dependent to a large extent on the presence of complement.[53] Nonspecific opsonic agents exist and these include non-antibody proteins such as globulin[115] and fibrin. Knisely has emphasized the importance of a fibrinlike coating of blood proteins on inert particles such as india ink and kaolin for their phagocytosis.[58]

The enviromental factors which affect phagocytosis have been neglected to a large extent. This is undoubtedly because tests of phagocytosis have been made in glass tubes or on smooth

surfaces of glass slides or cover slips. It was only rather recently that Wood and his associates began intensive investigations of enviromental aspects which affect phagocytosis.[93,115, 117, 118, 119] Wood has stressed the point that, where as maximum protein is necessary for phagocytosis in liquid media, when phagocytes act on a suitable rough surface, they may be successful withlittle help from serum globulins. Wood showed that even capsulated pneumococci (except type III) could be taken up readily by leukocytes on filter paper or membranes of fibrin. The walls of pulmonary alveoli similarly appear to offer a surface which is appropriate for phagocytosis in the absence of opsonins. The concept of surface phagocytosis may possibly more closely stimulate those conditions seen in vivo.

Thus far in the discussion on phagocytosis the types of cells involved and the effect of various factors upon particles as well as the effect of enviroment has been reviewed. One question still remains. What determines whether a particle is to be phagocytized or not? Inother words if these cells are to perform their function successfully, there must clearly be a reliable mechanism by which they can discriminate between foreign matter and dead tissue cells on the one hand and healthy tissue cells on the other. Elassical Theories have ascribed this discrimination to phenomena associated with surface energy and electrostatic charge.[8, 35, 73,77] Boyden feels that differences in surface charge could not possibly account for the high degree of selectivity characteristic of the phagocytic process. This high degree of selectivity was noted by Cameron (1932) when he saw the amoebocytes of a given species of earthworm will ingest spermatozoa of other earthworm species, but not spermatozoa from the same species.[18]

Boyden suggests that part of this successful discrimination maybe explained by the role of opsonins. If opsonins are indeed"natural AB", their interaction with antigen could cause the release of "chemotactic substance." The effect of the chemotactic substance principle is to draw the leukocyte close to the particle, phagocytosis may be viewed as the conclusion to this process. This theory is substantiated by the fact that the mammlian polymorphonuclear leukocyte is apparently incapable of recongnizing foreign matter in the absence of serum. The response of this cell to bacteria and other micro-organisms is dependent upon a reaction between serum recognition factors (opsonins) with foreign particles or substances. Boyden notes that in many cases the opsonized particle adheres firmly to the cell surface before it is phagocytized. It is possible that the affinity of the opsonized bacterium for the polymorphonuclear leukocyte is similar in mechanism to the affinity which antigen-antibody complexes have, in presence of complement, for primate red cell or non-primate platelets, (the immune adherence phenomenon Nelson 1963.)[75] There is, however, no uniform agreement upon the way a phagocyte distinguishes foreigness from non-foreigness.

Although much investigation has been done and is at present being done concerning the process of inflammation, certain fundamental principles are still discernable. It is these basic vascular, lymphatic and cellular changes which have been reviewed. Particular emphasis has been placed on chemotaxis and phagocytosis. In these areas emphasis has been directed to establishing basic facts as well as development of a hypothesis which might be applicable to the experimental work which will be presented.

II. The immune response and its similarity to inflammation

A. The possible presence of natural antibodies

The events which follow injection of foreign matter (bacterial, cellular or otherwise) into the skin of an immunized animal closely resemble those which follow a similar acute injury to the skin of a normal animal. We have already described the sequence of events in acute inflammation. Similarly in the immune reaction one of the first events which occurs is an increase in capillary permeability. In the case of the immune animal this reinjection of a foreign protein has been called cutaneous anaphylaxis.[12] The similarity of the immune response to acute inflammation continues as in the next phase large numbers of polymorphonuclear leukocytes become adherent to the walls of the vessels and migrate into the site of the injection.[59] As a result of a change in the capillary endothelial lining some edema of the connective tissue occurs. The reaction described reaches its maximum within a few hours. When this response is observed in a previously actively immunized animal it has been called the Arthus phenomenon.[3]

There appears to be a very close relationship between the sequence of events as described for the immune response and that described specifically for the acute inflammation. If one looks upon the inflammatory stimulus (bacterial, parasitic, etc) as a possible antigen stimulus and if natural antibodies exist for that particular antigen, then the acute inflammatory response would be very similar to or identical with the immune response.[12]

The theory of the existence and function of natural antibodies allows a method whereby the acute inflammatory response, the immune response and the recognition of self vs. non-self may be united under a common mechanism. Natural antibodies against heterologous

red cells have been demonstrated in the sera of many vertebrates including reptiles and amphibia.[105, 106] Boyden notes that there is no evidence that the positive reaction given by high concentrations of normal sera in serological tests are basically any different from the reactions of antisera in much higher dilution. In his experience he noted that the so called non-specific reactions of normal sera in passive hemagglutination tests are specifically inhibited by mixing antigen with the normal serum before adding the antigen coated red cells.[12] This fact would be in support of the possible presence of natural antibodies. The fact the natural antibodies are often seen _in vitro_ means that antigen-antibody complexes might be formed following injection of any antigenic substance. Antigen-antibody complexes may in turn initiate acute inflammation. There therefore, exists the possibility that the reaction of natural antibodies with the inflammatory antigenic stimulus could result in a natural antibody-antigen combination that could explain the inflammatory response observed.

This theory of the existence of potential performed antibody specific for each and every antigen has been proposed by Jerne. He postulates that somewhere in embryonic life there occurs a spontaneous production of globulin molecules which are possessed with a great varienty of random specificities. As the organism develops the globulin molecules which are reactive with the host components are absorbed out before the self-replicating mechanism comes into play. Those globulin molecules which are not absorbed are replicated. This replication process is greatly increased for a molecule of any specificity if the antigen is added.[55] In the case of bacteria and other common antigenic stimuli, it is possible to envision that natural antibodies do develop and subsequently,

when antigen concentration (bacterial conc.) becomes great enough, the antigen-antibody combination sets off an inflammatory response.

B. The possible reaction of antigen with cellular components

It has often been assumed that the antigen-antibody complexes which are formed in the tissues of immunized animals cause a certain amount of cell injury. Pharmacologically active substances are though to be released from the cells damaged by antigen-antibody interation and the sequence of events in the acute inflammatory reaction follows. An alternative possibility exists. Injury to cells initiates the formation of antigen-antibody complexes with cellular antigenic components. These complexes would in turn set into motion humoral events with the possible involvement of factors such as complement.[12]

Thus far we have been dealing with inflammatory situations in which there existed an antigenic substance. The question which must be raised is whether or not there is any immunological explanation for the inflammatory reaction of physical trauma. Inflammation occurs, but can the theory of immunological damage through natural antibody and antigen interaction be applied? It has been shown by Waksman that it is possible to create experimental immunization with damaged autologous and homologous cells.[108] It may be necessary to inject adjuvant in order to induce this response. Recent work by Thomas; however, showed anti-lung antibodies to be present in the patients with primary atypical pneumonia.[104] Waksman has also observed that in diseases associated with a considerable amount of damage that there exist autoantibodies to components of the damaged tissue.[108] The inflammation produced by a physical change may also be dependent upon antigen-antibody interaction. Boyden suggests injury of cells

causes liberation of the intracellular components. These components are, in turn, regarded by the host as antigenic and will react with the corresponding natural antibodies present in the body fluids.[12] Antigen-antibody complexes will be formed and the characteristic sequence of inflammatory events will follow.

The immune response as we think of it usually involves the interaction of antigen-antibody in an immunized animal. The sequence of reactions which are seen in this situation resembles closely those events seen in the acute inflammation. The presence of natural antibodies to certain cellular components and to antigenic substances such as bacteria has been demonstrated. It is, therefore, possible to postulate an antigen-antibody mechanism for inflammatory systems which are dependent upon specific antigen as well as physical injury to cell. The reaction of natural antibody with an antigenic substance such as bacteria or a component of the injured cell such as the basement membrane can result in the formation of natural antibody-antigen complexes., and these complexes can initiate the inflammatory response.

III. Review of work done in this laboratory

As was mentioned previously acute inflammation has been examined in various ways. The systems which have been utilized to examine joint inflammation have been varied, a sumarrized in an article by Gardner.[38] Recently Hollingsworth has examined the reaction of the suprapatellar bursae of the rabbit following the injection of foreign materials.

A. Why the knee joint?

In examination of the knee joint Hollingsworth suggests several reasons why this site is advantageous to use for inflammatory studies.

The technique is quickly mastered, knee joints come in pairs, comparison of results from one knee joint of an animal with the joint on the other side. In this way unresponsive animals are sometimes able to be ruled out of an experiment. The rabbit is convenient and has been widely used in studies of inflammation and immunity.[50] The synovium which lines the joint cavity is composed of loose vascular connective tissue. Recent studies of Suter and Majno concerning the structure of these capillaries indicates that they have an unusual fenestrated structure,[98] This could possible be related to the rapidity with which materials are exchanged by the synovium. As is known from anatomical and physiologic studies on diarthroidial joints, the synovial lining also provides the secretion of fluid for lubrication of the joints of the cavity. Synovial cells also remove foreign material from the cavity by phagocytosis.[41]

The method (which willbe examined in more detail in Section II of this thesis) consists of injection of the foreign material (undiluted or dissolved in 0.5 ml of 0.9% NaCl) into the suprapatellar bursae. The exudate produced can be examined by washing the bursae with saline, so that total cell exudate and cell morphology may be evaluated.

B. Results from the experiments

Hollingsworth has evaluated the response of injection of foreign materials into the rabbit suprapatellar bursae, both in the normal and the immune animals. One of the systems investigated was the reaction to ovalbumin. It was noted that ovalbumin, along with human albumin, human globulin, and bovine albumin, produced definite inflammatory responses six hours after intrasynovial injection. The effect of injection of the protein into animals

immunized against the protein was noted and it was, in general, seen that the immunized animals reacted with enhanced inflammation.[50] More recently the reaction to bacterial endotoxin (0.0005 ug) has been examined.[49] This inflammation resembles that produced by ovalbumin except that generally a relatively small amount of endotoxin (0.0005 ug) is required to produce an inflammation comparable to much greater quantities of ovalbumin (5 mg).

C. What are the possible mechanisms?

One may ask why some foreign substances which were examined produce an acute inflammatory response while others do not produce inflammation. Physical damage of the membrane by an unnatural (acidic or basic pHc) substance with possible contamination by endotoxin could lead to the development of inflammation. These possibilities were generally excluded because the materials were selected to avoid contamination and were of biological origin so that the inflammation produced was not likely based on their initial chemical nature.[70]

In immunized animals, the inflammation produced in the suprapatellar bursae resembles closely the Arthus phenomenon, in which the inflammatory cellular reaction is possibly a result of the complexes produced by antigen-antibody reaction.[3] Therefore the immune inflammatory response can be explained in terms similar to the explanation of the Arthus phenomenon.

In the previous sections some discussion was devoted to the similarity between immune inflammation and the acute inflammatory response. It was concluded that if natural antibodies existed to the foreign material which initiates the inflammation then the possibility exists for natural antibody-antigen complexes to cause the inflammatory response. It is possible that the same reasoning

can be applied to inflammation produced by ovalbumin and possibly, to a lesser extent, to that produced by endotoxin. The response seen with ovalbumin and endotoxin may be related to the fact that natural antibodies form complexes with them and thereby initiate the inflammation. Other possible explanations for the inflammation induced by ovalbumin and endotoxin exists. Ovalbumin may be associated with an increase in chemotaxis and subsequently phagocytosis will be increased.[12] Endotoxin may cause instability of the lysosomes (subcellular particles), which can cause cell damage and thus stimulate inflammation.[118] The evidence for both of these is rather ill defined. A more detailed treatment of hypothesis concerning the possible mechanisms of action of ovalbumin and endotoxin will be undertaken in part IV of this paper.

IV, Anti-inflammatory agents and their effect upon inflammation induced in the suprapatellar bursa of rabbits

Since both endotoxin and ovalbumin produced a predictable experimental inflammatory response in the knee joint, this system provided an excellent opportunity to examine the effect of pre-treatment with anti-inflammatory compounds. Sharp showed that certain anti-arthritic compounds--sodium salicylate, chloroquine, phosphate, phenylbutazone, and sodium aurothiomalte--decreased the premeability of the synovial membrane as measured by the removal of phenosulphophthalein (PSP), dye from the synovial cavity.[90] It was suggested by Sharp that all these anti-arthritic compounds decrease permeability and are thus anti-inflammatory. Sharp's article included specific drug schedules, and it was decided to determine the effects of drugs on exudation of polymorphonuclear leukocytes in the synovial cavity of the rabbit. Synovial inflammation was to

be induced by three different mechanisms: 1) The injection of foreign protein, ovalbumin (5mg) 2) Injection of ovalbumin (0.5mg) into the synovium of pre-immunized animals (local Arthus phenomenon) and 3) Injection of endotoxin (0.0005 ug). Preliminary experiments had demonstrated the nature of the cellular inflammatory exudate in the rabbit supra-patellar bursae using these methods. The purpose of using different anti-inflammatory compounds in these three types of inflammation was to delineate difference in effectiveness of drugs in different basic inflammatory processes. The drugs studied were chloroquine phosphate, phenylbutazone, cortisone acetate, and sodium salicylate with the treatment of saline acting as a control group.

Methods

General --- Male and female New Zealand white rabbits weighing 3-4 kg were used in all experiments. Individual animals were treated with one of four different drugs in a schedule outlined below with the saline group acting as controls. There were a total of 25 animals for each inflammatory system giving 5(animals) x2 (no joints) = 10 results for each drug in each of the three systems. Following drug treatment inflammation was induced in the supra-patellar bursa of rabbits with ovalbumin (5mg)[1] or endotoxin (0.0005 ug)[2]. Six hours following the inflammatory injection the joint capsule was washed with saline and number of inflammatory cells and their type was determined. In one experiment the animals were immunized with ovalbumin prior to drug treatment and the inflammatory dose was (0.5 mg) of ovalbumin.

Dosage Schedule[3]

Doses and Dilutions:

1) Phenylbutazone[4] (isotonic saline dilution)-----16mg/kg---ip. 7 days
2) Cortisone[5] (no dilution)--------------7mg/kg-------ip. 7 days
3) Sodium Salicylate[6] (no dilution)-------100mg/kg-------ip. 7 days
4) Chloroquine phosphate[7] (isotonic saline dilution)--8mg/kg--ip. 14 days
5) Sodium Chloride[8] ---------------0.9%-------ip. 14 days

(ip.---intraperitoneal)

1) Worthington Biochem. Corp., Freehold, N.J., U. S. A.
2) Organo Lab. Limited, Brettenham House, London, England
3) Taken from G W. C. Sharp[90]
4) Ohne Lab, New York, New York
5) Premo Pharmaceutical Laboratories Inc., South Hackensack, N.J. U.S.A.
6) Eli-Lilly Co., Indianapolis, U. S. A.
7) Winthrop Lab., New York, N. Y., U. S.A.
8) Cutter Lab., Berkeley, California or Chattanooga, Tenn., U. S. A.

The calculation of drug dosage could be related to human dosage as follows:

Compouhd	Approx human dose (mg)	Dose of 70 kg man (mg/kg	Rabbit dose (mg/kg)	Rabbit dose(mg/kg)	Ratio of Rabbit/ Man/ dose/ day
Sodium Salicylate	4000/day	57	300/day	100	approx. 2
Chloroquine Phosphate	250/day	3.5	35/day	8	approx. 2
Phsnylbutazone	600/day	8.5	50/day	16	approx. 2
Cortisone	250/day	3.6	21/day	7	approx. 2

The cortisone dosage was changed from intraperitoneal subcutaneous following experiment 3. The dosage was as follows: Exp. 4A --70mg-3days-sc; 4B--50mg-4days sc; 4C--25mg-7days sc; 5--25mg-7days sc; 6--25mg-5days sc; and 25mg solv-cortefon on fifth day.

Inflammatory Stimulus--- The knee joints of the animals were shaved with standard animal clippers. Animals were anestized by intravenous injection into the marginal ear vein with 5-7ml. of Pentobarbital sodium (20 mg/kg).[9] The suprapatellar bursae were injected with ovalbumin (5mg or 0.5 mg) or endotoxin (0.0005 ug) dissolved in 0.5 ml. of isotonic saline. Injection was done using 2cc syringes and a 21 gauge needle.

Harvesting of the Inflammatory cells---Six hourse following injection of the suprapatellar bursae the animals were killed with Pentobarbital Sodium (100 mg/kg). Two ml. of isotonic saline was injected into the suprapateller bursa with a "19" needle. The fluid was washed in and out of the joint approximately three times in order to remove as many of the inflammatory leukocytes as possible. The fluid was then removed, total volume was read and white blood counts were done

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(9.) Diamond Lab., Des Moines, Iowa, U. S. A.

using a Coulter Counter (Model A--Series 1819). A close correlation was established between machine counts and those done by hand. The inflammatory exudate was centrifuged at 3,500 RPM for thirty minutes, The supernatant was removed and to the remaining cells were added two drops of normal rabbit serum. This mixture was then brushed with a camel hair brush on to #00 coverslips. Differential counts were then determined following Wright staining. The total number of white blood cells could be determined as shown below:

WBC x 10^6 x no. ml. = total number of WBC

Immunization with ovalbumin--- Immunization was done using a mixture of equal portions of 1% ovalbumin and Freunds adjuvant. One ml. was injected into each of the four extremities (20mg total ovalbumin). Three weeks following the initial injection the animals were rechallenged using the same procedure.

Quantitative evaluation of Ab---One ml. of serum was mixed with 0.1 cc of ovalbumin (1mg/ml). This was mixed well and incubated at 37C for 30 minutes. Following incubation the mixture was centrifuged at 0° C and 3.500 RPM for thirty minutes. The supernatant was decanted and the precipitate at the bottom of the tube was read. To the supernatant of the tubes containing antibody and additional 0.1 cc of the previously mentioned ovalbumin (1mg.ml) was added and the procedure was repeated until no more precipitating antibody was present.

Results

In the introductory section it was stated that the effects of certain anti-inflammatory compounds - sodium salicylate, chloroquine phosphate, cortisone, and phenylbutazone - upon inflammatory changes induced in the synovial cavity (suprapatellar-bursa) of the rabbit would be investigated. The exudation of polymorphonuclear leukocytes into the synovial cavity of rabbits was induced by three different mechanisms: 1) Injection of foreign protein, ovalbumin (5mg) 2) Injection of endotoxin (0.0005 ug). 3) Injection of ovalbumin (0.5 mg) into the synovium of pre-immunized animals (local Arthus phenomanon) . There were originally only three experiments and the results are show below (Figures 1, 2, and 3).

Fig. I

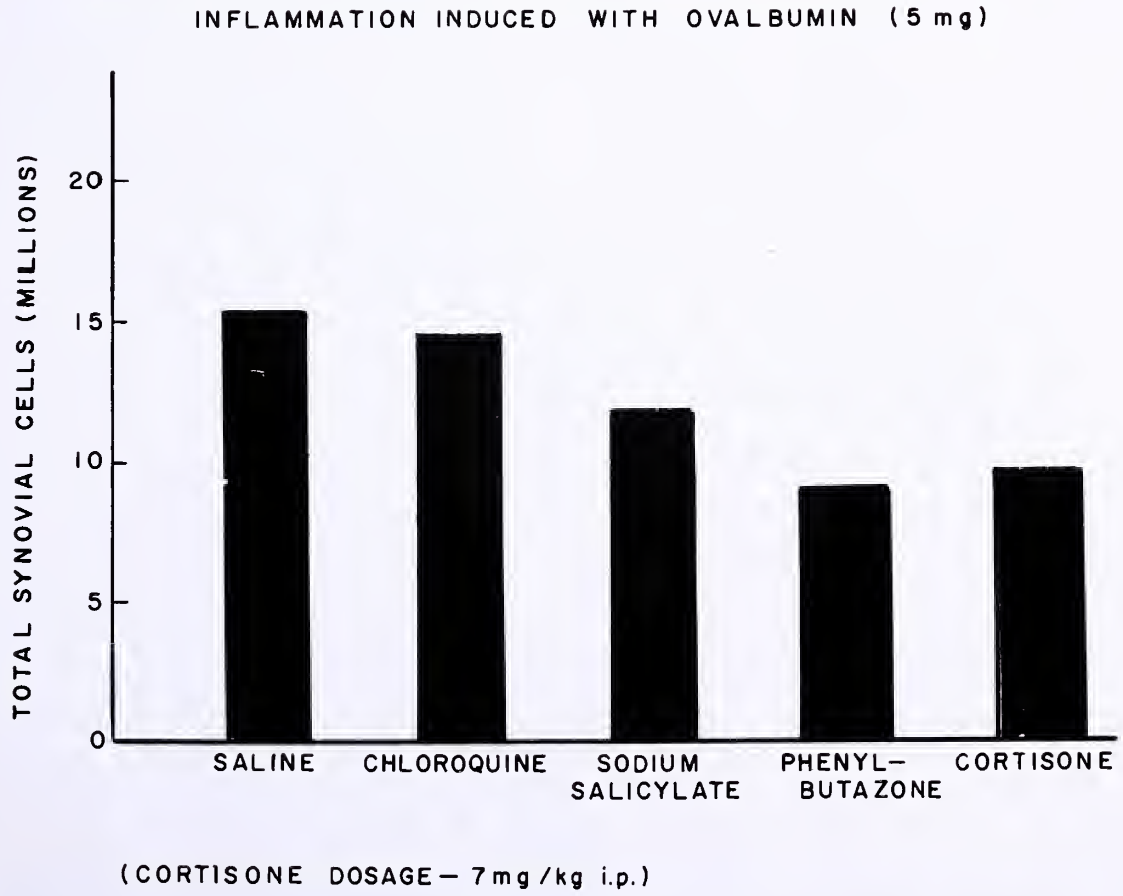


Fig. II

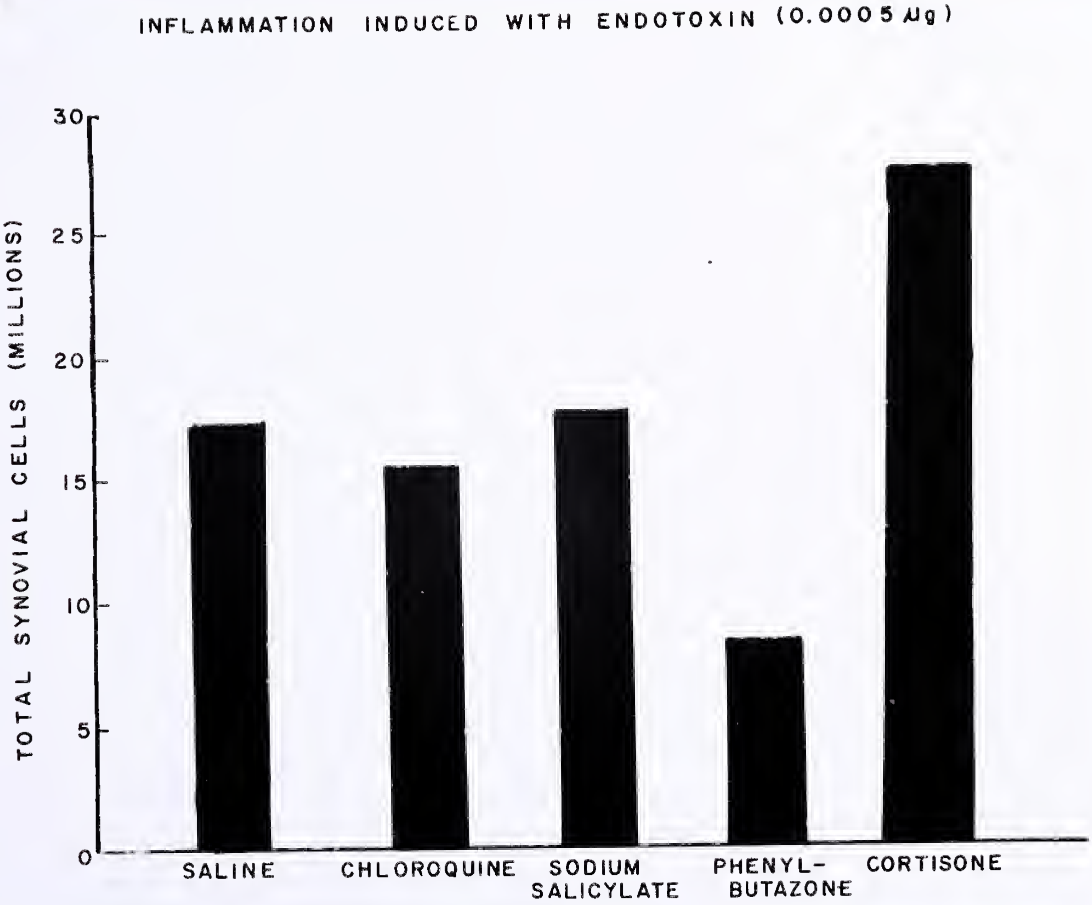
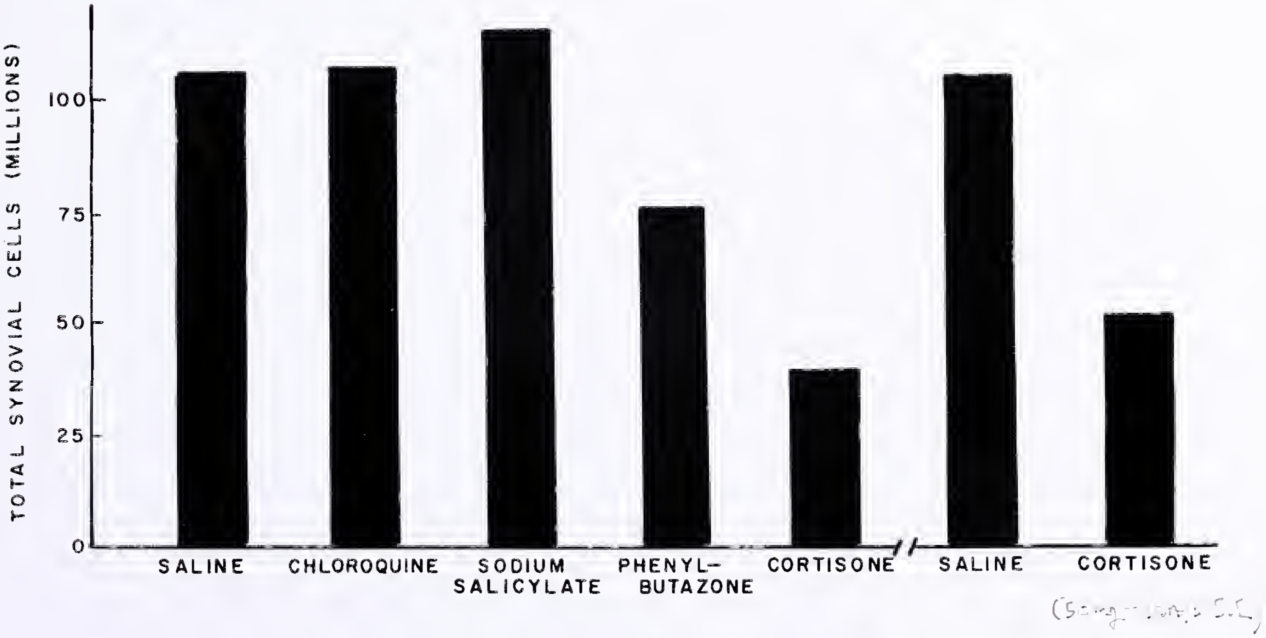

INFLAMMATION INDUCED WITH ENDOTOXIN (0.0005 µg)
TOTAL SYNOVIAL CELLS (MILLIONS)
30
25
20
15
10
5
0
SALINE
CHLOROQUINE
SODIUM SALICYLATE
PHENYL-BUTAZONE
CORTISONE

Fig III

INFLAMMATION INDUCED IN IMMUNIZED ANIMALS WITH OVALBUMIN (0.5 mg)

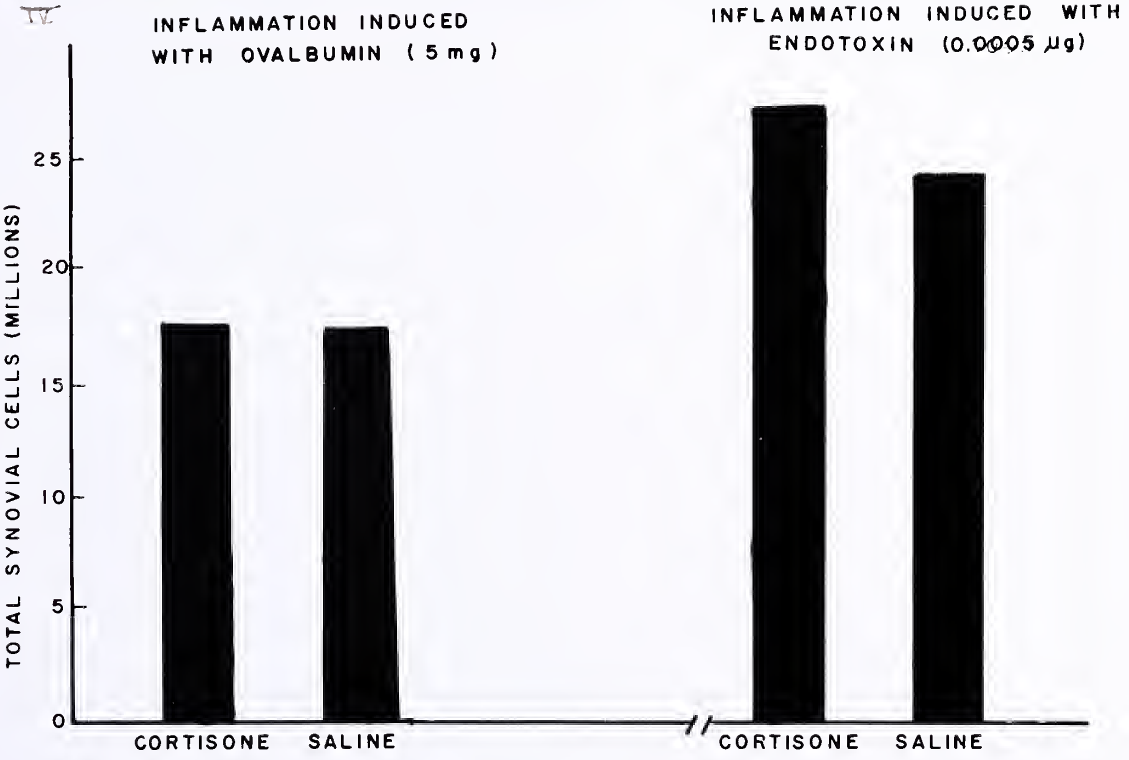


The cortisone dosage and route of administration was changed following the first three experiments. This was done because Frankel and Havenhill[37] showed that intraperitoneal administration of cortisone was only 20% as effective as the subcutaneous route of administration. The immunized animals were rechallenged administering cortisone subcutaneously. (Right hand portion of graph of Fig. 3)

The effect exerted by cortisone was questionable. In order to evaluate this effect animals were given cortisone subcutaneous in doses specified previously. In experiment 4A, B, C the right joint of the animals was injected with ovalbumin (5mg) and the left joint was injected with endotoxin (0.0005 ug). It was therefore possible to obtain a direct comparison of these two different systems and each of the animals was able to serve as its own control. It was noted in these experiments that cortisone did not stastically effect ovalbumin endotoxin inflammation. (Graph of Exp. 4C seen in Fig. 4)

A subsequent experiment using only endotoxin (0.0005 ug) as the inflammatory stimulus was done, (Fig. 5). Following cortisone treatment peripheral white blood counts and differentials revealed a relatively stable white count with a relative increase in the number of granulocytes and a decrease in the number of lymphocytes. This effect was expected if the animals were receiving an adequate dose of cortisone.

Exp. 4c
F → IV



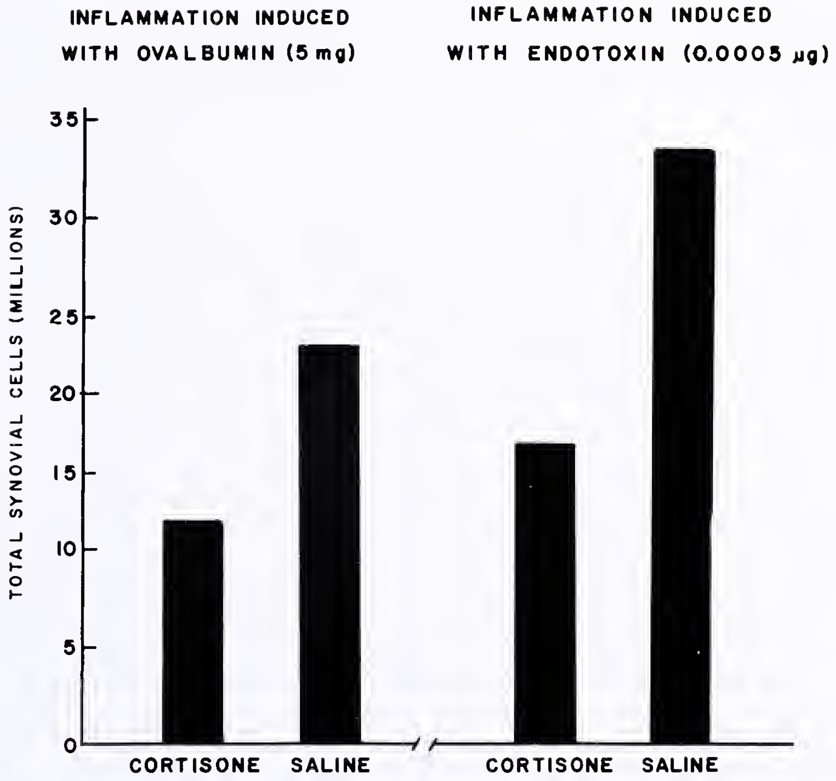
CORTISONE DOSAGE – 25 mg – 7 DAYS S.C.

FIG V

INFLAMMATION INDUCED WITH ENDOTOXIN (0.0005 µg).

In order to insure that adequate blood levels of steriod were attained 25 mg of cortisone acetate was given subcutaneously for five days and onthe final day of cortisone acetate treatment 25 mgs of solu-cortef (hydrocortisone) was injected intraveously. The animals were challenged by both endotoxin and ovalbumin was previously mentioned in experiment four. (Graph Fig. 6 below)

Fig II



CORTISONE DOSAGE—25 mg—5 DAYS S.C.
PLUS 25 mg SOLU-CORTEF (HYDROCORTISONE)

Conclusions

(1) Chloroquine phosphate, in this dosage schedule, had little or no effect on the inflammator systems. The values averaged very close to those of the sodium chloride controls.

(2) Sodium salicylate, in this dosage schedule, had little or no effect on these inflammatory systems. The values averaged very close to those of the sodium chloride controls.

(3) Phenylbutazone does have a marked effect upon the three inflammatory systems ($p \leq 0.05$) except in experiment I where $0.10 > p > 0.05$. It reduces the number of inflammatory cells significantly below that of the saline controls.

(4) Cortisone acetate had no stastistically significant effect upon the inflammation when given in doses specified in Exp. 4A, B, C (Fig. 4) and Fig. 5.

(5) Steroids decreased the inflammation produced by endotoxin and ovalbumin by stastistically significant levels when steroid levels were maintained by the administration of solu-cortef (hydrocortisone) immediately prior to challenge.

Discussion

I. Introductory remarks

The general conclusions which were reached from examination of the results have been stated. The results showed that chloroquine phosphate and sodium salicylate had little or no effect on the types of inflammation sutdied, and that phenylbutazone caused a marked decrease in the number of inflammatory cells present. When steroids were in adequate levels they appeared to reduce the inflammation in all situations.

In any animal experimental situations in which an attempt is made to evaluate the possible effectiveness of a drug, certain fundamental problems are presented. The route of administration should be such as to assure effectiveness. It has been noted previously by Sharp that the routes of adminstration which were used in this paper were effective in decreasing the permeability of the synovial membrane.[90] There was some difficult with the route of administration of cortisone acetate. Because of the work by Frankel and his collaborators the route of administration of cortisone acetate was changed from intraperitoneal route of administration was only about 20% as effective as the subcutaneous route of administration.[37] Steps must also be taken to be sure that the effect seen by the drug is not due to an effect upon a central mechanism such as a depression of bone marrow. During these experiments periodic white blood counts showed that the animals on drug treatment did not differ markedly from the saline control group. The final problem which is important to evaluate is whether or not any side effects exist in the animals and to what extent they may affect the experimental results observed. The only side effect which was observed in any of the animals treated was a slight water retention following cortisone therapy. A lymphopenia

and granulocytosis were also observed. These effects indicated that the cortisone dose was causing a well known hemotological change in the animals and was at a proper dose level to anticipate an anti-inflammatory effect. In summary it was felt that the animals which were drug treated did not have side effects or other manifestations which affected the experimental results, and that the dosage level was adequate to insure a response if the drug was effective.

By reviewing the anti-inflammatory properties of each drug one can more readily evauate the response which they have exerted in these experimental inflammations. It is also hoped that through analysis of the possible mechanisms of action of each drug that one can possibly arrive at a unified theory as to the possible mechanism by which ovalbumin and endotoxin are able to produce an inflammation.

II. Chloroquine phosphate

A. Mechanism of action for anti-inflammatory effects.

Chloroquine phosphate was first applied to the treatment of malaria but since that time many investigators have examined whether or not it is a valuable anti-inflammatory agent for use in such diseases as rheumatoid arthritis.[31, 32, 39] Sharp from his experimental studies feels that chloroquine is an effective anti-inflammatory agent.[90] Chloroquine has a prolonged period of unset[78] and this might be a key to the mechanism of chloroquine action. Bagnall suggest that it acts correct an in balance of some physiologic mechanism, such as one or more of the enzyme systems which affect the cells throughout the body. One such mechanism has to do with ATP (adenosine triphosphate.) It has been suggested that rheumatoid arthritis is associated with high effort level of the tissues, especially for ATP and a simultaneous lack of hormonal support.[45] It was further thought that inhibition of the tissues requirement for ATP might bring amelioration of many

of the symptoms of rheumatoid arthritis. Substances in the quinacrine group, of which chloroquine is a member, were found to have inhibitory effects on ATPase activity.[44] The mechanism of action of chloroquine might possible be related to an increase in ATP due to inhibitory effects on ATP ase activity. Recent work does not substantiate this and no other possible mechanisms have bee proposed.

B. Application to inflammation to the suprapatellar bursae.

From the review of chloroquine one can make the following conclusions which are applicable to the experimental results presented. Chloroquine seems to have a rather non-specific mode of action in reducing inflammation is such conditions as rheumatoid arthritis and that it is necessary to have a rather long period of treatment, greater than six weeks, before adequate therapeutic response is noted in humans. Sharp noted in his paper on evaluation of the permeability of synovial membrane that chloroquine has some effect upon drcreasing permeability but that this effect is not as great as that exerted by sodium salicylate and phenylbutazone. These two facts would lead one to suspect thatchloroquine may exert a minimal effect upon inhibition of the acute inflammatory response which isinduced by ovalbumin and endotoxin. No stastistically significant difference was noted between the numberof inflammatory cells in the saline treated animals in those animals treated with chloroquine for fourteen days. A revkin of the literature was compatible with this since it was noted that the action of chloroquine was rather slow in unset and thatthe value of chloroquine seemed to be in the management of chronic inflammatory conditions rather than the acute inflammation. Because little is known about the mechanism of action of chloroquine and sin e it did not exert any effect on the inflammatory systems examined, chloroquine provided no information about a possible mechanism by which

ovalbumin and endotoxin induced an inflammatory response in the suprapatellar bursae of the rabbit.

III. Sodium Salicylate

A. Mechanism of action of anti-inflammatory effects

Salicylates affect many physiological systems. Possible theories for salicylate mechanism of anti-inflammatory action can be best related to these physiologic systems.

Onesuch system is the effect salicylates have on the adrenal gland. Salicylate has been found to increase the production of 17-ketosteroids in the guinea pig. Since the adrenocortical hormones and ACTH were known to have anti-rheumatic effects there was considerable speculation that the anti-rheumatic effects of salicylates may be mediated through the adrenal system. There was also speculation that salicylates might act on the adrenal through the hypothalamus and pituitary. Therefore the "hypothalamic-pituitary-adrenal system" became a way of possible explaining salicylate anti-inflammatory effect. Much work showed similarities between metabolic and anti-inflammatory effects of salicylates and adrenocortical hormones or ACTH, but other have indicated that salicylates and corticoids have effects which are actually opposing.[5, 28, 92, 94] There are several points against an adrenal dependent theory. First is that several of the effects of salicylates are unlike those produced by corticoids. For example there is a reduction in hyperglycemia and glycosuria produced by salicylates in diabetic human subjects which is different than the effect of corticoids. Secondly, 17-ketosteroids have not been shown to be elevated under normal doses of salicylate.[39] It therefore seems certain that if antirheumatic effects of salicylates are in any way dependent upon intervention of the pituitary adrenal system, they are not dependent upon the maintenance of elevated circulating levels

of corticoids.[29]

Another physiologic system upon which salicylates exert their effect is on metabolism. Salicylates can increase O2 consumption and uncoupleoxidative phosphorylation. Salicylates also inhibited the formation of high energy phosphate bonds and this lead to many other metabolic changes. Adams and Cobb directed attention to the fact that certain non-hormonal antirheumatic (anti-inflammatory) drugs, like salicylate and phenylbutazone, uncouple oxidative phosphorylation.[1] Whitehouse[113] in turn noted that this selective uncoupling of oxidative phosphorylation inhibits I) the incorporation of inorganic phosphate into organic phosphates. II) the incorporation of organic sulphates into polysaccharide sulphates by bovine cartilage in vitro. It therefore appears that oxidative phosphorylation is uncoupled by salicylates in the connective tissue as well as in muscle, kidney, liver and other tissues. Whitehouse concluded by saying that there might exist some relationship between the ability to uncouple phosphorylation and clinical antirheumatic activity.[10] Subsequent work showed that salicylates, phenylbutazone, oxyphenbutazone, hydrocortisone, cinchophene, glycyrrhetic acid, fluflennic acid and 2.4 dinitrophen inhibited the metabolism of cartilage and other connective tissue in vitro, and also inhibited the biosynthesis of polysaccharide sulphates by rat cartilage in vivo. This drug action on cartilage metabolism could not be attributed to competition by the drug for available "active sulphate". Several of these drugs also depressed the excretion of sulphate ester in the urine. It was concluded that the later drugs uncouple oxidative phosphorylation in the whole animal both in peripheral tissues such as cartilage and in those visceral tissues such as kidney and liver, which are concerned with the biosynthesis of sulphate ester. These finding added further support to the concept that the anti-inflammatory drugs owe their property of suppressing the

inflammatory response, in part at least, to an ability to deprive the irritated tissue of its normal supply of energy in the form of ATP derived from cellular metabolism. Certain other agents such as steroids and chloroquine may also be effective in this way since they have been known to inhibit oxidative reactions.[114] One point against this theory for salicylates is that expressed by Done.[29] He points out that while these uncoupling effects are not produced by compounds which are devoid of antirheumatic properties, they are likewise not produced by many of the structural analogs of salicylates which are antirheumatic.

Salicylates can also exert an effect on enzyme systems. Bollet showed that sodium salicylate inhibited the transaminse which synthesizes glucosamine -6 phosphate from fructose-6-phosphate and glutamine.[76] The enzyme which synthesizes glucosamine-6-phosphate is the first of this sequence involved in the metabolism ofuridine diphosphoacetylglucosamine, UDPAG, which serves as a donor of acetylglucosamine moiety in the synthesis of hyaluronic acid. UDPAG may be converted into uridine diphosphoacetylgalactosamine, an intermediate in the synthesis of the galactosamine containing mucopolysaccharides, such as the chondrotin sulfates. The synthesis of glucosamine-6-phosphate is thus an initial step in the synthesis of both glucosamine and galactosamine containing mucopolysaccharides and an influence on the activity of this enzyme might result in significant alteration in mucopolysaccharide metabolism. In view of this evidence for the possible suppression of mucopolysaccharide synthesis by salicylate, it is pertinent to ask whether an overproduction of mucopolysaccharides occurs as part of the inflammatory process. Evidence is meager but some increase in concentration of mucopolysaccharides in the serum and increased excretion in the urine has been found in rheumatoid inflammatory disorders, in part at least, to an ability to regulate the [illegible] supply of energy in the form of [illegible] derived from cellular [illegible]. [illegible] steroids and [illegible] have been shown to inhibit [illegible] reactions. [illegible] that theory for salicylates [illegible]. [illegible] and other agents [illegible] which are [illegible] induced by [illegible] antirheumatic.

Salicylates can also exert an effect on [illegible]. [illegible] showed that sodium salicylate [illegible] glucosamine [illegible]. The enzyme which [illegible] this sequence [illegible] glucosamine, [illegible] which serves as a source of [illegible] in the synthesis of [illegible]. [illegible] into [illegible] of the [illegible] such as the chondroitin sulfates. The synthesis of [illegible] is thus an initial step in the synthesis of such [illegible] activity of [illegible]. [illegible] possible [illegible] it is [illegible] and [illegible] arthritis and systemic lupus erythematosus.[9]

The only othersystem which is affected by salicylate and may also be related to the possible mechanism of salicylate anti-inflammatory effect is the immunological response. Coburn et al did an experiment in which guinea pigs were injected with egg albumin intraarticularly and this was followed by intracardial administration of anti-egg albumin rabbit serum. This created a swelling of the joint due to the passive Arthus phenomena. Subsequent evaluation of the effect of drugs on this system was made. Inhibition was obtained only with agents which are therapeutically anti-rheumatic (sodium salicylate, aspirin gentisate, resorcylate, antipyrine, aminopyrine. phenacetin and p-aminophenol) but not with therapeutically inactive structural analogs.[20] With all of the other immunological systems, such as anaphylaxis, the drug distinction was not as clearly defined and did not coincide with the therapeutic value of the drugs. It is not clear whether the inhibitory effect of the salicylates and their analogues is due to their effect on the immunological process or whether the anti-inflammatory effect is dependent upon a physiologic phenomenon such as an increased permeability of the blood vessels which would cause diminished swelling.

Two other explanations have been made for the antirheumatic effect exerted by salicylates. The first of these depends upon the inhibition of hyaluronidase. When india ink or Evans blue dye was injected with hyaluronidase into the skin of rabbits or human subjects following the administration of sodium salicylate, Guerra observed decreased spreading as compared with normal controls.[81] He concluded that hyaluronidase in inhibited by sodium salicylate and that this activity is responsible for the therapeutic efficacy of this drug in the rheumatic state. Since this initial description it has been

confirmed by some[30, 79, 80, 88] and denied by others.[10, 15, 56] It seems to be that salicylates inhibit hyaluronidase <u>in vitro</u> only in enormously high concentrations and the evidence indicates that inhibitation is due not to salicylate itself but to minute amounts of oxidative products present as impurities.

The other explanation depends upon the fibrinolysin system. Evidence accumulated to suggest that fibrinolysin may play an important role in the development of inflammation, Therefore, the possibility that anti-inflammatory substances may inhibit fibrinolysin has been investigated.[107] In this system, much like the immunological one, certain of the anti-rheumatic salicylates seems to inhibit the system where others had no effect. There is therefore no convincing parallelism between this <u>in vitro</u> effect and antirheumatic properties.

Several mechanisms of actions have been proposed from the anti-inflammatory, antirheumatic effect exerted by salicylates. These have dealt with the pituitary adrenal system, metabolic systems including enzymens; the immunological response, hyaluronidase and fibrinolysin. One can only conclude that a drug which affects so many physiological systems must have many mechanisms of action and these may be related to those mentioned.

B. <u>Application to inflammation in the suprapatellar bursae</u>

Salicylates are rapidly absorbed by almost any route of administration including intraperitoneal injection in the rabbit. It has also been shown that the concentration of salicylate in the blood and in the joint fluid are similar.[89] There should be no difficulty in assuring anadequate level of salicylate in the animals. Salicylates appear tobe very active drugs in they affect many physiological systems. These effects on physiological systems may in turn be the mechanism by which salicylates exert their anti-inflammatory

effects. The systems and mechanisms which could possible apply to the acute inflammation induced in this project are basically three. Salicylates may affect the adrenal gland to cause an increase in production of corticoids which would in turn be anti-inflammatory. There are, however, arguments against this as mentioned previously. Salicylate may affect metabolism to uncouple oxidative phosphorylation[1, 113]. In this way salicylates can concurrently deprive the tissue of its normal supply of energy in the form of ATP derived from cellular metabolism and decrease the inflammatory response. Salicylates also inhibit certain immunological responses. As an illustration of this is the inhibition of the passive Arthus phenomenon as examined by Coburn and Haninger.[20] There are other possible theories for the anti-inflammatory mechanism which include inhibition of specific enzymes, fibrinolysin and hyaluronidase. Although these could also apply to the inflammation produced in the supra-patellar bursa of the rabbit the emphasis has been placed upon the three major theories.

Salicylates were shown by Sharp to be effective agents in decreasing the premeability of the synovial membrane.[90] One would predict from this work and the review of the mutiple effects of salicylate upon physiological system that salicylates might possible utilize one or all of the three mentioned mechanisms to produce an anti-inflammatory effect upon the inflammation induced by ovalbumin (5mg) and endotoxin (0.005ug). This was not the case. There was no stastically significant difference ($p < 0.05$) in any of the inflammatory systems between the number of white blood cells seen in saline treated animals and the number of white blood cells in sodium salicylate treated animals.

An effect might also have been expected in the immune animals where the system has close resemblence to that work done in guinea pigs

by Coburn and Haninger.[20] No statistical significant difference was seen between the saline and sodium salicylate groups. In actuality the results should not be compared for there were only two values for sodium salicylate.

At the present time no good factual information exists as to why no response was seen upon adminstration of sodium salicylate in these systems. One could postulate that the systems which were examined were acute inflammation whereas salicylates are generally most effective chronic inflammatory situations. An illustration of this is rheumatoid arthritis where salicylates have a greater chance to exert their metabolic, enzymatic and immunological effects than in a very acute inflammation. Salicylates do not stastically decrease the inflammation produced by ovalbumin and endotoxin and do not affect the immunological dependent inflammation. Because of this little can be said to relate the mechanisms of salicylates to the possible mechanisms that are responsible for the inflammatory response in the suprapatellar bursae.

IV. Phenylbutazone

A. Mechanism of action for anti-inflammatory effects

Very little work has been done on possible mechanism of action of phenylbutazone. In the earlier work it as postulated that phenylbutazone exerted some adrenotrophic effect. This has since been discarded.[27] More recently Whitehouse has shown that phenylbutazone inhibits the incorporation of s35 into cartilage mucopolysaccharides.[112] This he feels can be related to an overall uncoupling of oxidative phosphorylation. Phenylbutazone may possibly act as an anti-inflammatory agent by uncoupling oxidative phosphorylation and thereby inhibiting the acute inflammatory response.

B. Application to inflammation in the suprapatellar bursae

It is noted that phenylbutazone can exert four basic properties: analgesic, antipyretic, uricosuric and anti-inflammatory.[17] The anti-inflammatory property is the only factor which is important in affecting the inflammation induced in the suprapatellar bursae of rabbits. Phenylbutazone has a marked effect upon the three inflammatory systems examined ($p < 0.05$) except in experiment I (Fig. 1) where $0.10 > p > 0.05$. Consistently it reduced the number of inflammatory cells below that of the saline controls in all cases.

The fact that phenylbutazone was very effective in these active inflammatory states was not surprising since it is often used clinically in the management of acute inflammatory conditions such as thrombophlebitis, as well as in the management of the more chronic type of inflammation such as rheumatoid arthritis. Very little is known about the actual mechanism whereby phenylbutazone is able to exert its anti-iflammatory effect. There is some speculation that it possesses the ability touncouple oxidative phosphorylation and thereby inhibiting the aucte inflammatory response. This, however, does not provide any additional information which might be valuable in postulating a possible mechanism whereby ovalbumin and endotoxin are able to produce inflammation.

V. Cortisone

E. Mechanism of action of anti-inflammatory effects

For many years it has been known that steroids are valuable in the treatment of acute inflammation. The rational for their use in the treatment of inflammatory conditions like rheumatoid arthritis is worth examining since it might suggest possible mechanisms of action. Perhaps a sufficient rational for the use of steroids in these diseases is to prevent the permanent residual damage which often results from the acute inflammatory stage(s) characteristic of rheumatic and collagen

disease are in some way different from normal as far as adrenal function goes. Although adrenal insufficiency does not seem to exist there is considerable evidence that there are definite alterations of adrenal secretion and metabolism in these patients.[52, 57] At the present time these alterations seem to be a result (rather than related to the etiology) of the rheumatic and collagen diseases.

It has been suggested that since a general parallelism exists between the anti-inflammatory potency and interference with protein and carbohydrate metabolism, it is likely that the hormones may prevent the formation of cellualr elements and perhaps endogenous compounds which are important in the genesis of the inflammatory process. There is little proof to support this statement.

A possible explanation exists to explain the fact that cortisone tends to decrease excess connective tissue proliferation in response to inflammatory damage. Cortisone, along with salicylates, phenylbutazone and other drugs inhibited the metabolism of cartilage and other connective tissue *in vitro*.[10] Cortisone treatment also decreased the amount of S35 incorporated into both granuloma and normal connective tissue.[48] Decreased formation of connective tissue in response to inflammation may possible be explained if cortisone inhibits S35 uptake by connective tissue elements and also inhibits the metabolism of these elements.

Thomas and his associated have recently examined the effect of cortisone in differend experimental systems and from his work a theory which might be applicable to the experimental work presented in this thesis can be postulated. There observations also shed some light on the anti-inflammatory mechanism of cortisone.

In the late 1950's it was noted by Thomas and others that intravenous papain would temporary destroy the cartilage structure in the ear of the rabbit, resulting in temporary flopping of the ears. In 1960

Thomas and Fell noted that similar changes could be induced by experimental hypervitaminosis.A. They concluded from their observations that the changes seen in experimental hypervitaminosis A may be the result of activation of a proteolytic enzyme or enzymes with properties similar to papain.[101] Subsequently in 1961 they observed the effect of hydrocortisone on the response of the chick and mouse explants to excess vitamin A. In the presence of excess vitamin A, cartilage (chick, mouse) and bone (mouse) rapidly disintegrated, but when hydrocortisone was added to the medium, this dissolution of the intercellular material was much retarded, though not suppressed.[34] Subsequently in 1963 Thomas et al showed the depletion of cartilage matrix produced <u>in vivo</u> in rabbits by excess vitamin A palmitate could be largely prevented by concurrent administration of cortisone. This protective action of the adrenal corticoids seemed to be the direct effect on cartilage; in rabbits given an excess of vitamin A palmitate, the injection of hydrocortisone into one knee joint partially prevented the depletion of that articular cartilage matrix, as compared with the contralateral joint.[102] The effect which hypervitaminosis A had on cartilage was discovered to be due to the release of cell lysosomes. This work was done mainly by Dingle and his associates.[26, 102, 110] The action of enzymes released from the lysosomes was held responsible for the degradation of protein polysaccharide complexes in connective tissue. Weissman and Thomas suggested that effects of cortisone and hydrocortisone in antagonizing the effects of excess vitamin A was perhaps due to a stabilizing of lysosomes against the vitamin and other injurious agents. It was also shown that vitamin A induced the release of beta-glucuronidase and acid phosphatase from a granule fraction which contained lysosomes.[110] Corticostervins act directly to inhibit the release of lysosomal enzymes both <u>in vitro</u> and <u>in vivo</u>.

In summary it can be stated that cortisone possesses two properties which are important for its anti-inflammatory activity. The one property is the ability to prevent resynthesis or deposition of chondroitin sulfate in cartilage matrix and thereby altering the formation of functionally damaging connective tissue. The other property is the capacity to enhance the stability of lysosomes and thereby prevent the release of acid hydrolytic enzymes which can induce inflammatory cellular changes and cartilage damage.[103]

B. Application to inflammation in the supra-patellar bursae

It will be remembered that in experiment I (Fig. 1) and II (Fig.2) in which the inflammation was induced by ovalbumin (5mg) and endotoxin (0.0005 ug) that the route of administration of cortisone acetate was intraperitoneal. Subsequently it was discovered that this route of administration was approximately 20% as effective as the subcutaneous route of administration. For this reason the results seen in Experiment I and II were not examined. In the experiment in which ovalbumin (0.5mg) was injected into the bursa of previously immunized animals it was shown th t cortisone acetate markebly inhibited the inflammation. Since this experiment was dependent principally upon the interaction of antigen and antibody for the production of inflammation it was not surprising that cortisone was inhibitory. Cortisone has been shown to have decreased the antibody levels, decreased globulin, and also inhibited the Arthus phenomenon. One could only conclude that the effect which cortisone produces in this immunological dependent inflammation is related to one or possible to all of the factors mentioned above. Subsequently the effect of cortisone upon the inflammation induced by ovalbumin and endotoxin was investigated. The conclusions were that cortisone did not affect either type of inflammation. It was decided that the blood level of steroid might not be adequate to pro-

duce an effect. Another experiment was done in which 25mg of cortisone acetate was given subcutaneously for five days on the final day of cortisone acetate, 25 mg of solu-cortef (hydrocortisone) was injected intravenously. At the same time the animals were challenged with both ovalbumin (5 mg) and endotoxin (0.0005 ug) as in experiment IV (Fig.Iv). Both types of inflammation showed statistically significant reduction of the number of leukocytes when treated on this regiment of steroid, as compared to saline controls.

From review of the literature we can obtain some idea of how cortisone could act upon these inflammatory systems to decrease the number of white blood cells. The reason that cortisone reduces the inflammation produced by ovalbumin is not completely clear. This may be dependent upon its non-specific anti-inflammatory effect or it may be related to the fact that cortisone decreases immunological reactivity. This will be examined in detail in the section to follow. The work by Thomas and his collaborators give a possible clue as to how cortisone may act upon the acute inflammation produced by endotoxin. As mentioned previously cortisone and hydrocortisone have been shown to stabilize the lysosomes and thereby prevent the release of acid hydrolytic enzymes which can induce inflammatory cellular changes and cartilage destruction. Weissman and Thomas have demonstrated that promptly after the injection of aerobacter-aeoogenes endotoxin in young rabbits, there was significant increase in the release of two lysosomal enzymes from the large granular fraction of live homogenates prepared in sucrose.[111] Granular fraction from the liver of animals made "tolerant" to endotoxin and subsequently challenged with a single injection no longer responded in this manner. It was also noted that pretreatment with cortisone alone cause a definite, although modest, decrease the release of both enzymes, presumable due to the ability of cortisone to stabilize lysosomes. The inflammation which

is produced by endotoxin in the syprapatellar bursa may be dependent upon the release of two acid hydrolases from the lysosome of cells in the synovial cavity. Cortisone may possible inhibit this inflammation by increasing the atability of the lysosomes.

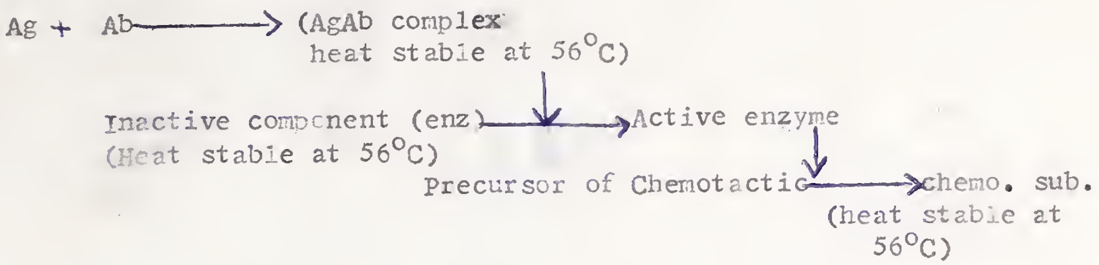
Proposed Mechanism by which Inflammation was Induced in the Supra-Patellar Bursa of Rabbits

In the introductory section of this paper significant discussion was given to the basic characteristics of inflammation with particular emphasis being given to chemotaxis and phagocytosis. Subsequently the immune response was reviewed and its similarity to inflammation was made.

In this thesis inflammation in the supra-patellar bursae of rabbits was produced by three different mechanisms: (1) Injection of foreign protein, ovalbumin (5mg) (2) Injection of ovalbumin (0.5 mg) into the synovium of pre-immunized animals (3) Injection of endotoxin (0.0005ug).

Perhaps the easiest inflammation to propose an explanation for is that presented by the injection of foreign protein ovalbumin (0.5mg) into the synovial bursa of pre-immunized animals. The inflammation is largely dependent upon combination of antigen and antibody. Boyden has investigated the chemotaxis which exerted by mixtures of antibodies and antigens. This was reviewed extensively in the introductory section of this thesis. The conclusions which were drawn were as follows: First, leukocytes are capable of responding to chemotactic stimulus by active directional migration in the presenceof inactivated as well as fresh serum. Secondly, the interaction of antigen-antibody results in the production of heat stable chemotactic substance (s). Thirdly, that the chemotactic substance is not produced when antigen-antibody are allowed to interact inheat inactivated serum.

This could be diagrammed below as is shown onpage 8.



If this is the actual mechanism by which antigen-antibody combination produce inflammation it could apply to the immune system examined in this thesis. Antibodies to ovalbumin could combine with the ovalbumin which is injected in the rabbit joint with subsequent production of "chemotactic substance"; the result would be white blood cell migration into the synovial cavity. This is purely theoretical at the present.

Two other possibilities exist as possible mechanisms for the production of the immune inflammation. Pharmacologically active substances may be released from cells which might be damaged by the interaction of antigen and antibody and the sequence of events in the acute inflammatory reaction follows. An alternative way of examining this exists. Boyden suggests that injury to cells may set in motion the formation of antigen-antibody complexes with the antigenic component being derived chiefly from the damaged cell components. He feels that these complexes would in turn set into motion humoralevents and possible involvement of such components as "chemotactic substance."[13]

A possible mechanism by which ovalbumin can produce inflammation in the normal animal is related to those already discussed. In the introductory section the possibility that natural antibodies may play a role in the production of inflammation was discussed. If natural antibodies existed to proteins which were phylogencially similar to those of the host and yet possessing their own specificity, the combination of natural antibody-antigenic protein could result in production of an inflammatory response. If natural antibodies existed to ovalbumin, an inflammatory response could be observed that was dependent upon the com-

bination of natural antibody to ovalbumin. This hypothesis could possibly be investigated with any foreign protein which gave a inflammatory reaction in the suprapatellar bursa by the use of immunological tolerance to that protein. The rabbit may possible be made tolerant to ovalbumin by injectionof large amount of ovalbumin during neonatal life. As the animal grows to maturity this tolerance could be maintained by frequent injections of ovalbumin. The animal would be unable to produce antibody to ovalbumin and no natural antibody against ovalbumin should exist. The animal could then be challenged by injection of the suprapatellar bursa. If the inflammation produced previously is dependent upon the presence of natural antibodies to ovalbumin then the response in the tolerant animals would be decreased below that of normal animals. At the present time this possibility is being examined.

In the discussion of cortisone considerable emphasis was placed on the fact that cortisone has been shown to stabilize lysosomes. This explains why endotoxin is able to produce inflammation in the suprapatellar bursa of rabbits. This system has been extensively studied by Hollingsworth and Atkins.[49]

It was noted in the discussion of cortisone acetate that endotoxin causes a release of two lysosomal enzymes from the large granular farction from liver homogenates prepared in sucrose.[111] It was also noted that animals made 'tolerant' to endotoxin and subsequently challenged with a single injection no longer responded as they did before by release of the lysosomal enzymes. Cortisone was also noted to cause a definite, although modest decrease in the release of both enzymes, presumably due to the ability of cortisone to stabilize lysosomes. It was postualted that inflammation which was observed by the injection of endotoxin in the suprapatellar bursa is dependent upon the release of

acid hydrolases from the lysosomes in the synovial cavity.

This theory and others were investigated by Hollingsworth and Atkins. If the above mentioned theory is true two facts should be investigated. First, 'tolerant' animals can be tested to see if their reactivity to endotoxin is dec eases as would be predicted since less lysosomal hydrolases should be present if there is a similarity to the work done by Weissman and Thomas.[111] Secondly, if some auti-inflammatory factor like hydrolase is released into the synovium it should be able to be detected.

Hollingsworth and Atkins observed no decrease in synovial inflammatory response in animals that are hyporeactive or 'tolerant' to other reactions to endotoxin. This is in contradiction to what would have been expected if the lysis of lysosomes was res onsible for the inflammation induced by endotoxin in the suprapatellar bursa.

Hollingsworth and Atkins also made attempts to demonstrate anti-inflammatory factors in serum or synovial washings after endotoxin injection. This was done partially because Atkins and Wood had demonstrated the appearance of endogenous fever-producing material (leukocyte pyrogen) after the introvenous injection of endotoxin.[4] They were also interested in whether the rupture of lysosomes and presence of hydrolase could be detected. In these experiments 0.0005 ug of proteus endotoxin was injected into both knees of donor rabbits. At intervals thereafter (1 minute, 15 minutes, 30 minutes, 2 hours and 6 hours), a donor was sacrificed, his bursa washed with 20ml of saline, the exudate cells were counted and the exudate centrifuged. The supernatant material (0.5 ml) was injected into the suprapatellar bursa of three recipient normals and their exudate was examined at 6 hours. It was noted at 15 minutes that donor bursal washings contained inflammatory material, persumable endotoxin, and the two hour donor exudate produced only mild inflammation comparable to that of saline.

From these data Hollingsworth and Atkins concluded that endotoxin seemed to be fixed to cells, degraded, or removed from the synovial cavity within two hours and no secondary autogenous inflammatory substance could be detected as the inflammatory reaction envolved in the bursa.

Hollingsworth and Atkins drew attention to the extreme sensitivity of the joint cavity to endotoxin. Six hours after injection of 0.00005 ug of endotoxin, the rabbit joints often yielded 10 million polymorphonuclear leukocytes, The relationship that this extreme sensitivity might have to human arthritis conditions was also briefly discussed.[49]

The proposed mechanisms for the explanation of the experimental production joint inflammation are quite theoretical. Ovalbumin may produce inflammation in the immune animal because of the ability of antigen and antibody to cause chemotaxis, or antigen-antibody complexes may cause cellular damage which will inturn cause inflammations. The production of inflammation by ovalbumin in the normal animal may be dependent upon the presence natural antibodies to ovalbumin. Endotoxin may possible produce its inflammation by the production of anti-inflammatory factors such as the release of acid hydrolases from lysosomes.

Appendix

Summary of Results

(Values expressed in millions of
White blood cells)

Experiment I---Inflammation iduced by Ovalbumin (5 mg) (Fig.I)

Chloroquine	Saline	Sodium Salicylate	Phenylbutazone	Cortisone
16.20	3.23	4.71	5.82	1.74
11.00	4.66	23.90	6.66	17.00
7.26	22.60	12.00	4.70	19.38
7.35	13.20	9.94	4,53	6.72
10.65	6.36	20.40	6.93	9.72
8.58	34.20	7.84	12.10	8.50
23.80	25.30	9.02	15.45	9.01
30.40	9.28	7.74	8.55	13.06
Ex=115.24	18.60	10.26	11.60	3.74
	137.43	105.81	12.80	88.87
			89.14	
Average 14.41	15.27	11.76	8.91	9.87

Experiment II---Inflammation enduced by endotoxin (0.0005ug) (Fig.II)

Chloroquine	Saline	Sodium Salicylate	Phenylbutazone	Cortisone
9.30	9.35	6.80	7.05	36.20
28.60	10.30	19.00	6.35	17.10
7.56	36.00	4.58	4.95	33.00
8.80	26.80	7.30	13.50	26.00
4.85	16.30	32.40	4.95	17.30
10.10	18.00	11.00	9.00	13.80
20.06	7.10	26.50	5.94	32.90
4.17	15.50	22.10	3.74	46.70
32.60	8.70	26.10	12.70	27.10
28.90	23.22	22.50	15.40	26.00
Ex154.94	171.27	178.28	83.58	276.10
Average 15.49	17.13	17.83	8.36	27.61

Experiment III---Inflammation induced in immunized animals with ovalbumin (0.5 mg)(Fig. III)

Saline	Chloroquine	Sodium Salicylate	Phenylbutazone	Cortisone
140.8	163.2	134.2	78.8	21.8
118.0	122.4	98.2	55.6	55.6
81.2	105.4	x=116.2	88.2	x=38.7
80.6	99.8		93.2	
93.2	93.2		x= 76.45	
122.4	58.0		Sig.	
x106.33	107.0			

Experiment III-B---

Saline	Cortisone	
163.2	42.7	
117.6	37.2	
53.2	70,9	
64.8	77.6	
100.6	35.7	
118.9	35.6	
85.2	29.6	
132.9	40.8	
x= 105.85	27.7	
	55.0	
	99.8	
	88.2	
	x= 53.4	Sig.

Experiments 4 A, B, C--Inflammation induced by ovalbumin in the right joint (5 mg) and Endotoxin into the left joint (0.0005 ug)

A.

Ovalbumin		Endotoxin	
Cortisone	Saline	Cortisone	Saline
12.6	18.0	55.8	27.2
7.12	5.6	33.6	68.0
9.00	6.8	x= 44,6	18.4
38.72	30.4		113.60
			x= 37.86

B.

Ovalbumin		Endotoxin	
Cortisone	Saline	Cortisone	Saline
16.9	30.6	14.8	27.5
27.7	14.5	29.4	13.5
19.8	13.5	23.9	27.4
26.2	23.1	24.2	15.0
x= 22.62	x=18.3	16.6	19.4
		24.1	x=20.36
		x= 22.16	

IV.

Experiment 4C Cortisone dose 25mg. 7days S.C. (Fig. IV)

WBC Day starting injections 10/21/64

WBC	Polys	Lymphs	Monos	Eosinophils
1) 9,900	25	50	24	1
2) 17,800	4	76	20	
3) 12,900	2	88	10	
4) Different animal				
5) 9,600	5	78	16	
6) 13,100	2	86	12	
7) 9,600	1	82	17	
8) 8,800	14	63	23	

WBC Last day of Cortisone treatment 10/28/64

WBC	Polys	Lymphs	Monos
1) 10,000	76/3	19	2
2) 8,000	73/3	17	7
3) 8,714	69	28	3
4) 6,098	76/1	16	7
5) 9,648	88	5	7
6) 11,024	69/2	22	7
7) 10,500	77/4	13	6
8) 9,578	85	10	5

Ovalbumin induced inflammation		Endotoxin induced inflammation	
Saline	Cortisone	Saline	Cortisone
32.4	19.5	41.2	27.6
25.3	13.9	13.8	33.0
15.2	18.7	18.4	30.8
16.7	30.4	20.2	36.6
10.8	14.6	26.2	44.4
11.1	23.2	35.0	26.6
14.2	11.0	15.7	12.5
17.2	8.8		7.3
x= 17.86	x= 17.51	x= 24.36	x= 27.35

No statistical significance was seen between the two values.

Experiment 5 (Fig. 5)

Endotoxin	
Cortisone	Saline
16.8	44.3
16.2	54.6
9.3	19.4
8.5	20.0
14.7	13.6
28.5	23.3
12.0	61.1
23.4	37.2
19.8	19.2
18.9	26.6
21.6	56.2
24.1	67.0
EX= 213.8	4.4
	22.5
x= 17.81	25.0
	51.2
	EX= 541.2
	x= 36.08

There was a stastistically significant difference $p < 0.05$ between the two values.

Experiment 5

Animal #1

Day	WBC	Polys	Lymps	Monos	Eosinophils
1	14,100	37	59	3	1
2	12,700	65	36	1	
3	11,200	78	19	4	
4	10,500	72	24	5	
5	12,700	82	16	2	
6	9,500	84	15	1	
7	11,000	88	12		

Animal #2

Day	WBC	Polys	Lymps	Monos	Eosinophils
1	9,900	37	56	6	1
2	9,000	52	43	6	
3	6,700	64	26	6	
4	5,000	70	26	4	
5	5,500	73	24	3	
6	4,600	87	11	2	
7	6,000	84	12	4	

Animal #3

Day	WBC	Polys	Lymph	Monos	Eosinophils
1	9,000	48	42	10	
2	11,000	79	19	4	
3	8,000	80	16	4	
4	11,600	84	12	4	
5	14,600	92	6	2	
6	10,000	92	8		
7	12,000	87	10	3	

Animal #4

Day	WBC	Polys	Lymph	Monos	Eosinophils
1	11,100	33	60	7	
2	13,400	51	39	10	
3	6,000	72	22	6	
4	5,500	84	16	0	
5	6,000	76	12	8	
6	5,700	80	13	11	
7	6,700	88	11	1	

Animal #5

Day	WBC	Polys	Lymph	Monos	Eosinophils
1	9,035	47	44	9	
2	10,700	79	18	3	
3	8,300	88	9	4	
4	11,200	88	8	4	
5	8,000	84	14	2	
6	8,500	87	12	1	
7	6,700	86	12	2	

Animal #6

Day	WBC	Polys	Lymph	Monos	Eosinophils
1	14,100	51	44	6	
2	9,000	79	17	4	
3	10,000	90	4	5	1
4	8,000	84	12	4	1
5	9,500	89	10	1	
6	9,000	90	9	1	
7	10,500	94	6		

VII.

Summary of the values

Experiment 6--Inflammation induced by ovalbumin (5mg) in the right joint and endotoxin (0.0005 ug) in the left joint.

	Ovalbumin		Endotoxin	
	Cortisone	Saline	Cortisone	Saline
	15.38	41.36	13.53	31.74
	17.26	11.97	28.31	46.62
	17.15	33.40	16.75	54,18
	8.75	29.04	11.19	42.00
	11.75	14.04	13.61	38.72
	8.79	23.00	10.31	21.24
	6.84	30.60	6.21	30.60
	5.58	9.52	3.78	33.30
	6.84	10.71	10.00	13.40
	17.11	34.44	42.70	39.06
	11.70	10.56	25.07	19.00
Total	127.15	248.81	181.46	368.86
N	11	11	11	11
Aug.	11.55	22.62	16.50	33.53

Cortisone Acetate
Dose- 25mg I.M. for 5 days 25mg solu-cortef 5th day
Challenge-5th day

Differential and White blood cell counts showed a moderate lymphocytopenia.

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